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The Harmala Alkaloids: Evidence for Their Complex Inhibition of the K^+-Acyl Phosphatase Reaction of Membranes

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SUMMARY

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Although the harmala alkaloids were originally reported to be specific inhibitors of the Na^+-activation site of (Na^+ + K^+)-ATPase, their reaction is now known to be more complex as the K^+-activation site of this enzyme is also thought to be involved. By using a fluorescent method of assay employing 3-O-methyl fluorescein phosphate as substrate, and by appropriate correction of the data for the fluorescence quenching of harmaline itself, we have confirmed that this alkaloid directly interacts with the K^+ activation site of the acyl-phosphatase partial reaction of (Na^+ + K^+)-ATPase. However, calculation of the Hill coefficients for harmaline inhibition of K^+-acyl phosphatase and for K^+-activation of the system in the presence of harmaline suggests that activation and inhibition do not occur at the same site.

INTRODUCTION

The harmala alkaloids are hallucinogenic compounds which induce disturbances in behavior and perception (1, 2). At the physiological level they have been shown to have a wide variety of effects upon intestinal transport (3), amino acid uptake (4), cellular cyclic nucleotide levels (5), monoamine oxidase activity (6, 7), acetylcholine production (8), membrane potential (9), and even the cardiovascular contractile force (10). Whether these alkaloids act by some related mechanisms, possibly by exerting their effects through the inhibition of a common intermediate, or by separate inhibitory processes is not known at present.

Recent studies by Canessa *et al.* (11) and by Charnock *et al.* (12) have shown that these alkaloids will inhibit membrane bound (Na^+ + K^+)-ATPase (EC 3.6.1.3.), thus leading to an alteration in the electrolyte distribution within many cells. These latter studies have focused attention upon the mechanism of inhibition of (Na^+ + K^+)-ATPase by these agents. For example, by measuring the effects of harmalol, harmine and harmaline on both the rate of substrate (ATP) hydrolysis and Na^+ stimulation of enzyme phosphorylation, Canessa *et al.* concluded that these alkaloids were specific competitive inhibitors of Na^+ binding to the enzyme (11). Subsequently Charnock *et al.* demonstrated that in addition to the interaction at the Na^+ site of the enzyme, harmaline would inhibit the binding of [^{3}H]ouabain to the enzyme both under conditions of optimal enzyme phosphorylation, that is in the presence of Mg^{++}, ATP and Na^+, or in the presence of Mg^{++} and Pi but in the absence of substrate (12). Kinetic analysis of their data also suggested that harmaline displayed a mixed competitive/

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non-competitive inhibition of ATP hydrolysis, as well as a direct interaction with Mg^{++} which occurred both in the presence of, and in the absence of, enzyme protein (12, 13).

A further example of the complex nature of the interaction between the harmala alkaloids and $(Na^+ + K^+)$-ATPase comes from the work of Robinson (14) who has reported that by using umbelliferone phosphate as the substrate, he could demonstrate harmaline inhibition of the K^+-activated acyl phosphatase reaction (EC 3.6.1.7) thought by many investigators to be the ouabain-sensitive partial reaction of $(Na^+ + K^+)$-ATPase. This latter inhibition was shown to involve a marked decrease in the affinity of the enzyme for K^+ in the presence of harmaline, thus suggesting a direct effect of harmaline upon the K^+-binding site of the enzyme.

In this paper we report further evidence to support the findings of Robinson, but suggest that the interaction of harmaline with K^+-acyl phosphatase may not necessarily occur at the same site at which K^+ activation occurs.

MATERIALS AND METHODS

Because the enzyme ouabain-sensitive K^+-acyl phosphatase (EC 3.6.1.7) is thought to be a partial-reaction of the $(Na^+ + K^+)$-ATPase (EC 3.6.1.3) of many mammalian cell membranes, we determined the activity of K^+-acyl phosphatase in the microsomal fraction of ox brain homogenates which had been enriched with $(Na^+ + K^+)$-ATPase by detergent extraction in the presence of ATP (15).

Acyl-phosphatase activity was determined using 3-O-methyl fluorescein phosphate (3-O-MFP) as substrate following a slight modification of the assay procedure described by Huang and Askari (16). The assay media contained 40 mM glycylglycine, 4 mM $MgSO_4$, 1 mM H_4EDTA adjusted to pH 7.6 by the addition of Tris. Concentrations of KCl and harmaline (HME) were varied as indicated in the text. After addition of 3.3 μM 3-O-MFP to the temperature equilibrated cuvette, the reaction was initiated by the addition of 10 μl aliquots of enzyme suspension. The final assay volume was 2 ml.

The reaction was followed at 37° in a Hitachi Perkin-Elmer Spectrophotofluorometer (Model MPF-4) fitted with a cuvette temperature-jacket controlled by an external Haake PK10 circulating water bath. The excitation wavelength was 470 nm and the emission wavelength was 512 nm. Increasing fluorescence intensity was monitored with time.

Protein concentrations were determined by the method of Lowry *et al.* (17) using bovine serum albumin (BSA) as the reference standard.

Hill plots (18) were analyzed by the method of Chou (19); the Hill coefficient for activation by K^+ is given by N_H where:

$$\log \frac{V}{V_{max} - V} = N_H \log K^+ - \log K_{0.5}$$

where K^+ is the activating ligand and V_{max} is the velocity obtained from the asymptote of V at maximal K^+, while the values for V were determined at fixed concentrations of the inhibitor (i.e., HME).

Conversely the value for M_H, the Hill coefficient for the inhibitor harmaline (HME), was obtained from

$$\log \frac{V_o - V_i}{V_i} = M_H \log HME - M_H \log I_{0.5}$$

where V_o and V_i are, respectively, the velocities in the absence and in the presence of the inhibitor, at fixed concentrations of the activating ligand K^+. The concentrations of $K_{0.5}$ for K^+ and $I_{0.5}$ for HME, were calculated from the least squares fit to the above equations.

Glycylglycine, harmaline, 3-O-methyl-fluorescein and 3-O-methyl fluorescein phosphate were all obtained from the Sigma Chemical Company.

Deoxycholate and other chemicals were of analytical reagent grade.

RESULTS

Because the harmala alkaloids are themselves fluorescent compounds, it was first necessary to establish the extent of any spectroscopic interaction between the inhibitor harmaline (HME) and either the fluorescent substrate 3-O-methyl-fluores-

cein-phosphate (3-O-MFP) or the product 3-O-methyl fluorescein (3-O-MF) formed by the reaction with K^+-acyl-phosphatase (ED 3.6.1.7). This is shown in Fig. 1 where the decrease in relative fluorescence intensity (F.I.) of 3-O-MF which occurred with increasing concentration of harmaline is illustrated. The mean increase in fluorescence intensity from five sequential additions of 20 μl aliquots of 3-O-MF (containing 0.23 nmoles) was used to calculate the change in relative fluorescence intensity ΔFI/nmole. In addition it can be seen from Fig. 2, that increased concentrations of HME quench the fluorescence of both the substrate (3-O-MFP) and the product (3-O-MF) to a similar degree. However, it should be noted that although all three compounds, that is HME, 3-O-MFP and 3-O-MF, fluoresce at 512 nm, the relative magnitude of their fluorescence at this wavelength is markedly different (1:520:11,000, respectively).

The effect of several concentrations of HME on the K^+ activation of the acyl-phosphatase reaction is shown in Fig. 3. In the top panel (A) the results are given as the variation in the maximal velocity of the reaction (V) as a function of the concentration of added K^+ at zero, 0.5 mM and 1 mM HME. In the lower panel (B) the same data are presented after correction for fluorescence quenching by harmaline. It can be seen that anomalous results would be obtained if uncorrected data were employed and fluorescence quenching was ignored. Obviously the extent of quenching increases with increasing concentration of HME.

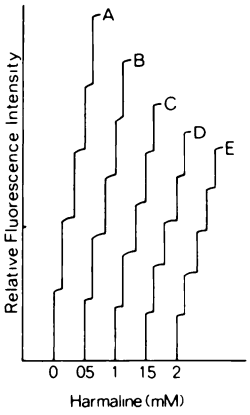

FIG. 1. *Relative fluorescence intensity of 3-O-methyl-fluorescein (3-O-MF) at various concentrations of harmaline (HME)*

Results from a typical experiment are shown. Scales are arbitrarily off-set to correct for the initial fluorescence intensity of harmaline.

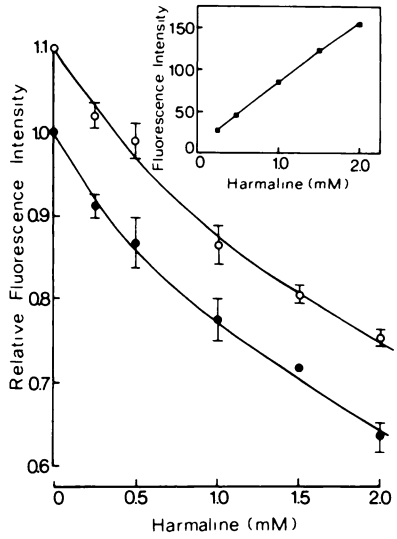

FIG. 2. *Fluorescence quenching of both substrate 3-O-MFP* (○) *and product 3-O-MF* (●) *of K^+-acyl phosphatase by harmaline.*

The results are expressed as the mean ± SE from three separate experiments with the relative fluorescence intensity (in the absence of harmaline) for 3-O-MFP and 3-O-MF set equal to 1 and 1.1, respectively. The unquenched relative fluorescence units for 1 μM 3-O-MFP was 60.6 ± 14.8, while that for 1 μM 3-O-MF was 1270 ± 59.4. The fluorescence intensity of increasing harmaline concentrations at a comparable instrument sensitivity is shown in the inset. Where no standard errors are shown, they lie within the symbol.

This is further illustrated by the double reciprocal plots of this data shown in Fig. 4. Here we have plotted the reciprocal of $\sqrt{v}$ against that of the ligand concentration, as recommended by several authors (20–22) for data where multiple enzyme activation sites exist. These illustrations suggest that harmaline is a competitive inhibitor of K^+ when the experimental data have been corrected for fluorescence quenching as in the lower panel (B) but one might have concluded that mixed competitive/non-competitive inhibition existed from the uncorrected data displayed in the upper panel (A).

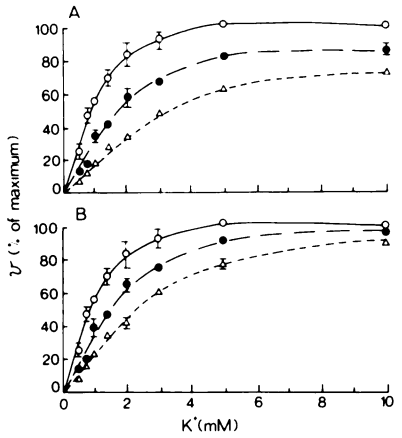

FIG. 3. *The effect of K^+ concentration on the rate of production of 3-O-MF in the presence of 0 mM* (○), *0.5 mM* (●) *and 1 mM* (△) *harmaline*

The maximum velocity, observed in the presence of 10 mM K^+ in the absence of harmaline, was arbitrarily set at 100. Other velocities, corrected for K^+-*insensitive* acyl phosphatase activity, were expressed as a % of this velocity. The results are the means ± SE of triplicate determinations from two separate experiments on different membrane enzyme preparations. Where no standard error bars are shown, they lie within the symbol. Panel A—Rates observed when the results are not corrected for fluorescence quenching by harmaline. Panel B—Rates when the results are corrected for fluorescence quenching by harmaline.

From a series of similar experiments with four separate membrane preparations with widely different specific activities of K^+-acyl-phosphatase, and again using data which had been corrected for fluorescence quenching, we calculated that the mean ± SD value for the concentration of K^+ required to produce one-half maximal increase in K^+-acyl-phosphatase activity, i.e. K_A, was 0.87 ± .05 mM. Under these conditions the mean ± SD inhibition constant for harmaline (K_I) when calculated by the method of Dixon (23) was 0.72 ± 0.17 mM. That is, although the V_{max} of these enzyme preparations varies from 1240–3200 nmoles 3-O-MF produced per mg protein/hr, the values for K_A and K_I do not change significantly. The individual values for each of these separate experiments are given in Table 1. The mean value for K_I of harmaline on K^+-acyl-phosphatase determined here is similar to those obtained for the effect of HME on the overall (Na^+ + K^+)-ATPase reaction in which concentrations of HME between 0.4 and 1.0 mM have been reported to give from 30–60% inhibition (11–13).

Our experimental procedure (outlined under METHODS) also allowed us to examine the effects of HME of the K^+-*insensitive*

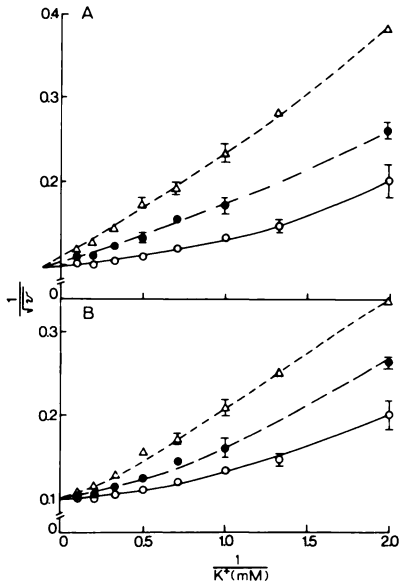

FIG. 4. *Double reciprocal plots of the K^+ concentration dependence on the rate of 3-O-methyl-fluorescein (3-O-MF) production*

Velocities shown in Fig. 3A and B were replotted as $\sqrt{v}$ as proposed by others when multiple activation sites are involved (20–22). Panel A—Rates when the velocities are not corrected for fluorescence quenching by harmaline. Panel B—Rates when the results are corrected for fluorescence quenching by harmaline. Where standard errors are not shown, they fall within the symbol. Note the different point of intersection of the lines shown in Panels A and B.

TABLE 1

Kinetic parameters of K^+-acyl phosphatase found in ox brain membrane preparations enriched with (Na^+ + K^+)-ATPase

Experiment	V_{max}[a]	K_A	K_I
1	1240	0.92	0.75
2	1530	0.80	0.50
3	2680	0.85	0.75
4	3200	0.90	0.90
Mean		0.87	0.72
± SD		0.05	0.17

[a] Expressed as Δ nmole 3-O-MF produced/mg protein/hr ± the addition of 10 mM K^+. K_A and K_I are defined in the text.

acyl-phosphatase present in these preparations. This activity never exceeded 5% of the total activity determined in the presence of optimal K^+. The mean value for $K_{I_{50}}$ under these conditions was 0.76 mM when calculated from data which again had been corrected for fluorescence quenching. That is, HME was also an inhibitor of an acyl-phosphatase reaction which did not require added K^+ ions for activation.

An alternative method of analysis of simple rate data can be obtained by employing the method originally developed by Hill (18). More recently Chou (19) has re-defined the set of equations which can be obtained from Hill plots, and which permit the calculation of several Hill constants. These equations were given under METHODS.

We therefore carried out several experiments with membrane preparations of K^+-acyl phosphatase which yielded data that was again corrected for fluorescence quenching, and from which we calculated the values for N_H and M_H for K^+ and HME, respectively. The mean values for these Hill coefficients as well as the related values for $K_{0.5}$ and $I_{0.5}$ for K^+ and HME are given in Table 2. Clearly the value for M_H for harmaline is 1, while the value for N_H for K^+ is 2. As previously demonstrated by Robinson (14), increasing concentrations of harmaline decreased the apparent affinity for K^+, while in addition our experiments show that increasing concentrations of K^+ decreased the apparent affinity for harmaline.

TABLE 2

Hill constants for K^+ and harmaline interaction with membrane bound acyl-phosphatase

K^+ constants* with variable concentrations of harmaline		
Harmaline	N_H	$K_{0.5}$
(mM)		
0	1.82 ± 0.11	0.80 ± 0.08
0.5	1.95 ± 0.11	1.25 ± 0.09
1.0	1.78 ± 0.06	1.66 ± 0.12
Harmaline constants* with variable concentrations of K^+		
K^+	M_H	$I_{0.5}$
(mM)		
0	0.99 ± 0.05	0.81 ± 0.05
0.75	1.21 ± 0.15	0.95 ± 0.11
2	1.19 ± 0.11	2.16 ± 0.53

* Hill coefficients were derived as described under METHODS. Results are expressed as the means ± SE from four determinations each on separate enzyme preparations.

DISCUSSION

While the work of Canessa *et al.* suggested that the harmala alkaloids were specific competitive inhibitors of Na^+ activation of (Na^+ + K^+)-ATPase (11), the more recent studies of Robinson indicated that by competing with K^+ ions for the activation site on the acyl-phosphatase partial reaction of the system, these hallucinogenic drugs inhibit (Na^+ + K^+)-ATPase by affecting both Na^+ and K^+ activation (14). In addition, previous work by Charnock *et al.* (12, 13) has also suggested that interaction between the alkaloids and either ATP or Mg^{++} ions may be involved in this inhibition, as well as other nonspecific effects upon protein.

In the present work we were able to confirm the inhibitory action of harmaline upon K^+-activated acyl phosphatase, as well as its inhibitory effect on some basal acyl-phosphatase which did not depend upon added K^+ for activity. These observations are qualitatively similar to our earlier observations of harmine inhibition of both sheep-kidney ouabain-sensitive (Na^+ + K^+)-ATPase and the ouabain-insensitive basal Mg^{++}-ATPase of these membrane preparations (12).

Because this present study and that of Robinson employed fluorescent substrates, inhibitors and products, it was important to determine the extent of any possible spectroscopic interaction between these agents. Our results demonstrate the value of correcting such data for the quenching of the fluorescence intensity of the reaction product by the inhibitor, for without this correction erroneous conclusions might be drawn.

While the results of Robinson on this point are not clear (see Fig. 4 of reference 14) they suggest to us that harmaline is a mixed competitive/non-competitive inhibitor for K^+ for K^+-acyl phosphatase, as did our own results before correction for fluorescence quenching. After correction our

data suggest that harmaline is a competitive inhibitor of K^+ activation of this enzyme activity thus confirming the general conclusion of Robinson rather than that of Canessa *et al.* (11).

Although comparison of our calculated value for the K_A of K^+ with other published values is difficult, since the K_A apparently depends upon the particular acyl-phosphate substrate employed (24), our value of 0.9 mM obtained with 3-O-MFP as substrate is in reasonable agreement with that obtained by Robinson using umbelliferone phosphate (14).

The Hill coefficient of 2 for K^+ is indicative of multiple site binding for K^+ with cooperative interaction between these sites—in agreement with many other published observations on this system—(25–27), while that of 1 for harmaline suggests that either a single site for harmaline binding affects one or both of the K^+ binding sites, or perhaps more probably that multiple sites for harmaline binding are independent of each other. The implication is clearly that the site of harmaline binding is not identical with that for K^+ binding.

While there is no direct evidence from the present experiments, the recent work of Madsen and co-workers with glycogen phosphorylase (28–30) has shown that allosteric binding can result in competitive inhibition of enzyme activators.

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